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From: **W. McCoy, J. Skinner, W. Ryan, Jr.**

Subject: **Massachusetts Compliance.**
CDC Method For Nicotine, Methodology Issues: Activity 7A14

Introduction

This memo has considered three areas of the CDC protocol that are of concern.[1] The first concern is with the preparation of certain critical solutions and the possible non-uniformity of the concentration of quinoline internal standard in these solutions. The second concern arises from the ambiguity in whether to adjust for dilution the nicotine concentrations used in the standard additions assay determination of recovery values. The third concern was what recovery factor to use to correct the final nicotine levels in the sample tobaccos. In order to maintain consistency, all samples were analyzed and corrected for recovery in the same manner.

Preparation of Solutions

As described in the CDC protocol, the stock internal standard (IS) solution is prepared by transferring 10.00 grams of quinoline to a 250-mL volumetric flask and diluting to volume with methyl tert-butyl ether (MTBE). This stock IS solution has a quinoline concentration of 40.0 mg/mL.

Following the CDC protocol, the stock standard nicotine solution is prepared by weighing 1.000 gram of nicotine and transferring to a 100-mL volumetric flask and diluting to volume with MTBE. It is noted that this stock standard nicotine solution has a nicotine concentration of 10 mg/mL and contains **no** quinoline internal standard (IS).

The CDC protocol further directs that the five working or dilute standard nicotine solutions are prepared by adding the indicated volumes of the stock standard nicotine solution and the stock IS solution to 50-mL volumetric flasks and making to volume with MTBE that contains **no** quinoline internal standard. Based on these volumes, each dilute nicotine standard solution contains the same amount of the quinoline internal standard (IS).

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Table 1.
Preparation of Dilute Standard Nicotine Solutions

Std No.	Volume Added Stock Nicotine Solution mL	Calculated Concentration Nicotine in Solution mg/mL	Volume Added Stock IS Solution mL	Calculated Concentration IS in Solution mg/mL
1	0.5	0.1	0.5	0.4
2	1.0	0.2	0.5	0.4
3	2.0	0.4	0.5	0.4
4	3.0	0.6	0.5	0.4
5	4.0	0.8	0.5	0.4

These five dilute standards are used to generate a calibration curve. The calibration data was fitted with a linear equation to the line:

$$Y = a + bX;$$

where: X = concentration of nicotine in mg/mL[1]
Y = $\text{Area}_{\text{Nicotine}} / \text{Area}_{\text{IS}}$
a = intercept on the ordinate (Y axis)
b = slope of the line

The Extraction Solution is prepared by pipetting 10 mL of the stock IS solution into a 1000-mL volumetric flask and diluting to volume with MTBE. This Extracting Solution has a quinoline concentration of 0.4 mg/mL. The CDC protocol assumes that both the dilute standards and the Extraction Solution will have the same 0.4 mg/mL concentration. In the opinion of Philip Morris analysts, the use of this single Extraction Solution to prepare both the stock and dilute standard nicotine solutions would remove any concerns with uniformity of the quinoline concentration in the different solutions.

Standards Addition Assay

The CDC protocol requires that prior to analyzing any samples, "*the testing facility must validate the system to verify that matrix bias is not occurring during nicotine extraction. This is done by analyzing the nicotine calibration standards in the same vegetable matrix*" as the samples. Into each of six culture tubes, 1.000 grams of tobacco were weighed and the volume of the 10 mg/mL stock standard nicotine solution shown in Table 2 was added. Note that this stock standard nicotine solution contains **no** quinoline internal standard.

[1] In the CDC procedure as printed in the Federal Registry, "X = concentration of nicotine in mg" This is assumed to be a typographic error and the nicotine concentration units are mg/mL.

Table 2.
Calibration Solutions for Standard Addition Assay

Sample	Tobacco Weight g	Volume Added Stock Nicotine Solution mL
Tobacco Blank	1.000	0.0
Tobacco Standard #1	1.000	0.55
Tobacco Standard #2	1.000	1.1
Tobacco Standard #3	1.000	2.2
Tobacco Standard #4	1.000	3.3
Tobacco Standard #5	1.000	4.4

After a 10-minute equilibration, the samples were then treated with 5 mL of 2N NaOH and 50 mL of Extraction Solution, MTBE containing 0.4 mg/mL quinoline as IS, and then analyzed by gas chromatography as described in the CDC protocol. Subtract the Area $_{Nicotine}$ / Area $_{IS}$ of the blank from the Area $_{Nicotine}$ / Area $_{IS}$ of each of the standards and fit a least squares line as described above.

One critical question is what nicotine concentration is used on the abscissa (x-axis). Consider the following text from the Standards Addition Assay Section (II.B.2.) of the CDC protocol.

> *"To prepare a nicotine standard corresponding to a concentration of 0.8 mg/mL, pipette exactly 4.4 mL of the nicotine standard (II.A.2.a) to one of the culture tubes. To obtain nicotine concentrations equivalent to 0.6, 0.4, 0.2, and 0.1 mg/mL, pipette precisely 3.3, 2.2, 1.1, and 0.55 mL, respectively, of the nicotine standard into four of the remaining culture tubes."*

This portion of the text would seem to indicate that the nominal concentrations (i.e. 0.8, 0.6, 0.4, 0.2, 0.1 mg/ml) are to be used for the abscissa (x-axis). Accordingly, Philip Morris analysts used the nominal concentrations in the Standards Addition Assay.

However, as shown in Table 3, the actual nicotine concentrations calculated from the weight of nicotine and the volumes of the Stock Nicotine and Extraction Solutions are greater than the nominal values. Nowhere in Section II.B.2 or elsewhere of the CDC protocol is there any indication that the nicotine concentrations are adjusted for the volume effects.

Table 3.
Actual Nicotine Concentrations in Standards Addition Assay

Volume Added Stock Nicotine Solution mL	Weight Added Nicotine mg	Volume added Extraction Solution mL	Total Volume mL	Concentration Nicotine in Solution mg/mL
0.55	5.5	50	50.55	0.1088
1.1	11.0	50	51.1	0.2153
2.2	22.0	50	52.2	0.4215
3.3	33.0	50	53.3	0.6191
4.4	44.0	50	54.4	0.8088

Similarly, as shown in Table 4, the actual quinoline concentrations calculated from the weight of quinoline and the volumes of the Stock Nicotine and Extraction Solutions are less than the 0.4 mg/mL concentration of quinoline in the dilute standard solutions, the Extraction Solution and the sample extract solutions. The $Area_{IS}$ value is directly proportional to the concentration of the quinoline and will effect the $Area_{Nicotine}$ / $Area_{IS}$ ratio. There is no adjustment for this dilution effect on the quinoline concentration in the CDC protocol.

Table 4.
Actual Quinoline Concentrations in Standards Addition Assay

Volume Added Extraction Solution mL	Weight Added Quinoline mg	Volume Added Stock Nicotine Solution mL	Total Volume mL	Concentration Quinoline in Solution mg/mL
50	20.0	0.55	50.55	0.396
50	20.0	1.1	51.1	0.391
50	20.0	2.2	52.2	0.383
50	20.0	3.3	53.3	0.375
50	20.0	4.4	54.4	0.368

Further questions with the Standards Addition Assay arise. Consider now the following text from the Section II.B.11 of the CDC protocol.

> *"Determine the recovery of nicotine by pipetting 10 mL of the 0.4 mg/mL nicotine standard to a screw capped tube containing 1.0 mL of 2 N NaOH and 10 mL of extraction solution (II.D.1). Cap the tube and tighten. Shake the contents vigorously and allow the phases to separate. Transfer an aliquot of the organic phase to an injection vial and inject."*

Philip Morris analysts used the pure nicotine 0.4 mg/mL standard for this portion of the Standards Addition Assay. However, ambiguity is not lacking here, it may be argued that the 0.4 mg/mL standard prepared in the tobacco matrix should be used because the CDC protocol is unclear as to what standard should be used.

Sample Analysis

Section II.B.12 represents another point for consideration.

> *"Compare the results of steps II.A.2. and II.B. If they differ by a factor of 10% or more, the recovery of nicotine from the aqueous matrix is not equivalent to recovery from the vegetable matrix of the ····· tobacco product. In this instance, the nicotine concentration of the ····· tobacco product must be determined from a nicotine calibration curve prepared from nicotine standards in a vegetable-based matrix."*

A recovery study using pure nicotine standards (II.A.2.)is compared with the recovery study using the vegetable or tobacco matrix standards (II.B.). As reported previously, the recovery from the aqueous matrix with the pure nicotine standards averaged 100.2%.[2] The Standards Addition Assay was run for all tobacco samples analyzed. The recoveries from the tobacco matrix ranged from 83.5% to 94.7% with ten recoveries above 90.0% and ten recoveries below 89.9%. For those 10 samples with recoveries below 89.9% the CDC method defines the recovery from the aqueous matrix is not equivalent to recovery from the tobacco matrix and the nicotine concentration of these samples must be determined from a nicotine calibration curve prepared from nicotine standards in a tobacco matrix. Philip Morris analysts used the following procedure. All tobacco samples were analyzed using the five working or dilute standard nicotine solutions prepared in MTBE and containing quinoline as internal standard (IS). These nicotine values were subsequently corrected for recovery using the results from the Standards Addition Assay conducted in the tobacco matrix. The recovery factor for each sample was individually determined using that material for the tobacco matrix. In order to maintain consistency, all samples were analyzed and corrected for recovery in the same manner.

Reference:

1. Federal Register, Vol. 62, No. 85, page 24115 - 24119, May 2, 1997.
2. W. McCoy, I. Skinner, W. Ryan, Jr., "Massachusetts Compliance. CDC Protocol For Nicotine, Status Report I: Activity 8A18," memo to Distribution, October 30, 1997.

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